JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 168–173 Vol. 43, No. 1
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.168–173.2005

Characterization of *ermB* Gene Transposition by Tn*1545* and Tn*917* in Macrolide-Resistant *Streptococcus pneumoniae* Isolates

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Received 14 August 2003/Returned for modification 8 October 2003/Accepted 24 September 2004

In *Streptococcus pneumoniae*, the *ermB* gene is carried by transposons, such as Tn*917* and Tn*1545*. This study investigated the relationship between macrolide resistance and the presence of the *ermB* gene on Tn*917* or Tn*1545* in 84 Japanese pneumococcal isolates. Macrolide-resistant strains were classified into two groups as follows. Group 1 (19 strains) showed a tendency to high resistance to erythromycin (MIC at which 50% of isolates are inhibited, 4 mg/liter; MIC at which 90% of isolates are inhibited [MIC_{90}], 128 mg/liter) but susceptibility to rokitamycin (MIC_{90}, 1 mg/liter), with the *ermB* gene located on Tn*1545*. Group 2 (65 strains) showed a tendency to high resistance to both antibiotics (MIC_{90}s for both erythromycin and rokitamycin, >128 mg/liter), with the *ermB* gene located on Tn*917*. There were no strains with constitutive macrolide resistance in either group. All of the strains in group 2 had a deletion in the promoter region of *ermB* and an insertion of the TAAA motif in the leader peptide. The results of pulsed-field gel electrophoresis and serogrouping showed that Tn*1545* spread clonally while Tn*917* spread both horizontally and clonally. In conclusion, in Japanese macrolide-resistant *S. pneumoniae* isolates, the *ermB* gene is carried and spread primarily by Tn*917*.

Streptococcus pneumoniae is still a major cause of respiratory tract infections, sinusitis, and acute otitis media. Antimicrobial resistance of *S. pneumoniae* has spread all over the world, and an increase in macrolide resistance has also been reported (1, 5, 7). In Japan, the macrolide resistance of *S. pneumoniae* has increased dramatically over the past decade and has reached a rate of 80% in clinical isolates, although there are some regional variations.

In *S. pneumoniae*, macrolide resistance is commonly caused by two major mechanisms: the efflux pump and target modification. The efflux pump, encoded by *mefA*, is associated only with resistance to 14- and 15-membered macrolides (19). On the other hand, target modification due to methylase, encoded by *ermB*, confers macrolide-lincosamide-streptogramin B (MLS_B) resistance (10). Among Japanese clinical isolates of *S. pneumoniae*, 42.7, 52.7, and 3.3% of strains have *mefA*, *ermB*, or both genes, respectively (6).

It has been reported that two transposons, Tn*1545* (22) and Tn*917* (17), carry the *ermB* gene in *S. pneumoniae*. Tn*1545* is almost identical to pAM77 from *Streptococcus sanguinis* (98% identity) and also mediates resistance to tetracycline via *tetM* (12) and resistance to kanamycin via *aphA-3* (4). In contrast, Tn*917* has no resistance genes for tetracycline or kanamycin and was identified on the nonconjugative multiple-resistance plasmid pAD2 in *Enterococcus faecalis* DS16 (17).

In this study, we investigated macrolide resistance among *S. pneumoniae* isolates in Japan with respect to (i) the relationship between transposon Tn*1545* or Tn*917* and resistance to macrolides, (ii) the question of whether macrolide resistance is inducible or constitutive, and (i) regional differences in the prevalence of the transposons in Japan.

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MATERIALS AND METHODS

Bacterial strains. Eighty-four macrolide-resistant *S. pneumoniae* strains were used in this study. These strains were initially selected by resistance to erythromycin and clindamycin (13). They were isolated between 1998 and 2003, at four hospitals and one laboratory in Japan, from the sputa of patients with lower respiratory tract infections and from the nasopharyngeal secretions or rhinorrhea of patients who had sinusitis with or without acute otitis media. The strains were preserved at our laboratory. All isolates were identified by their sensitivity to optochin and by the bile solubility test (16), as well as by PCR amplification of the *lytA* gene (8).

Antimicrobial agents. Reference powders of known potency of the following antimicrobial agents were used: penicillin G (Meiji Seika Kaisha, Ltd., Tokyo, Japan), erythromycin (Shionogi Pharmaceutical, Osaka, Japan), azithromycin (Pfizer Laboratories, Gorton, Conn.), rokitamycin (Asahi Kasei, Tokyo, Japan), and clindamycin (Upjohn, Tokyo, Japan). All of these antimicrobial agents were kind gifts from the respective manufacturers. Telithromycin (Nippon Hoechst Marion Roussel, Tokyo, Japan) was also used.

Determination of MICs. MICs were tested by using the six antibiotics mentioned above at concentrations between 0.06 and 128 mg/liter. MICs were determined by the twofold agar dilution method using sensitivity test agar (Mueller-Hinton agar medium; Eiken Chemicals, Tokyo, Japan) with 8% Strepto Haemo supplement (SHS; Eiken Chemicals). Bacteria were cultured overnight at 35°C in sensitivity test broth (Eiken Chemicals) supplemented with 8% SHS, after which the culture was diluted to a final concentration of 5×10^7 CFU/ml with buffered saline containing gelatin. The bacterial suspensions were then plated with an inoculator (Sakuma Seisaku, Tokyo, Japan) at an inoculum size of 5×10^4 CFU/spot onto agar plates containing various concentrations of a test drug. The plates were incubated for 18 h at 35°C, and the MIC was defined as the lowest drug concentration that prevented visible growth of bacteria.

Detection of resistance genes and analysis of the regulatory regions of the *ermB* gene. The presence of macrolide resistance genes was investigated by PCR using a commercially available kit (Gene Amp PCR kit with AmpliTaq DNA polymerase; Takara, Kyoto, Japan) and a model PH2000 DNA thermal cycler (Perkin-Elmer Cetus Instruments, Emeryville, Calif.). The primer set for the internal region of *ermB* comprised 5′-GAAAAGGTACTCAACCAAATA-3′ and 5′-AGTAACGGTACTTAAATTGTTTAC-3′, while that for the internal region of *mefA* comprised 5′-AGTATCATTAATCACTAGTGC-3′ and 5′-TTC

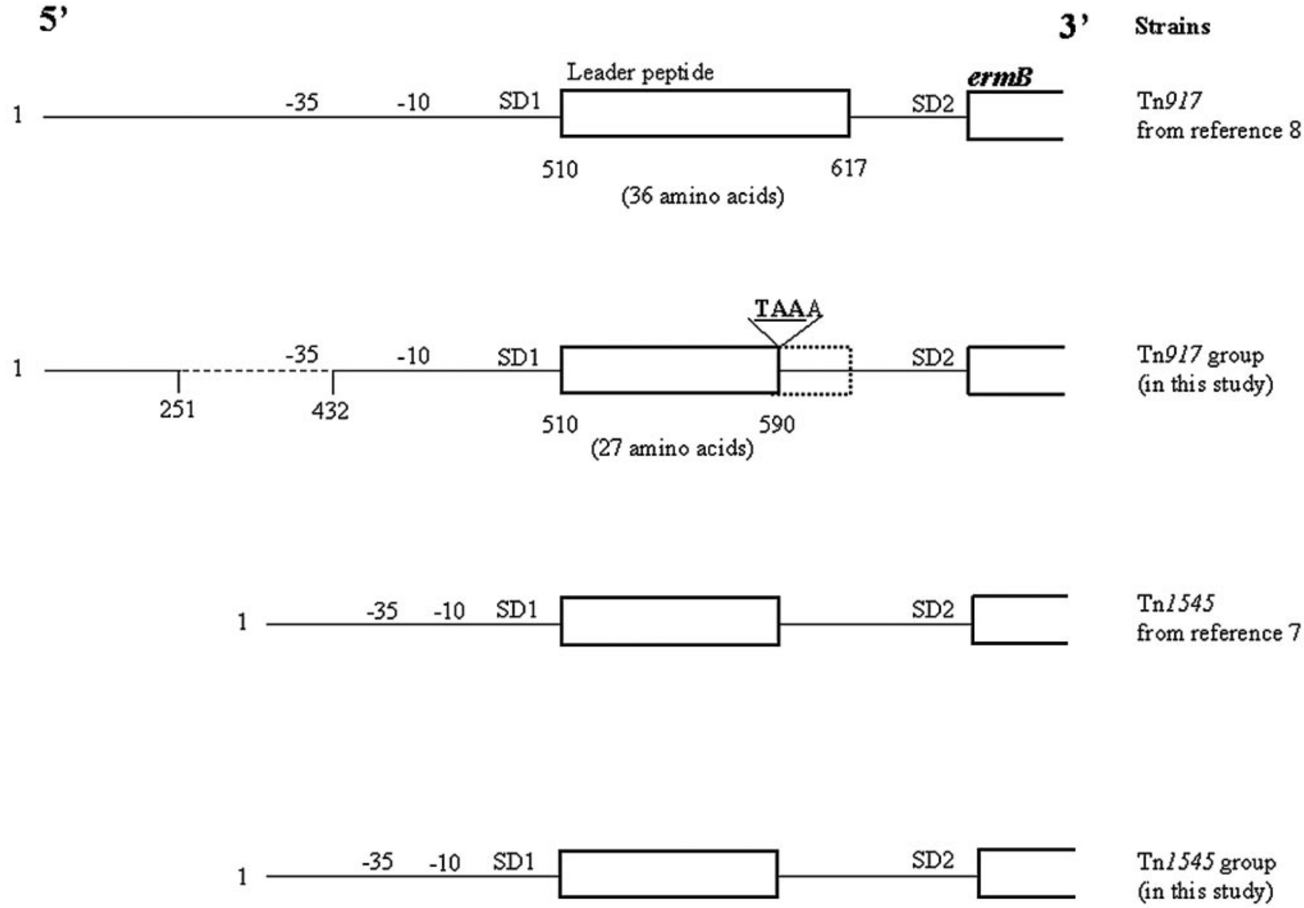

FIG. 1. Schematic representation of the reported regulatory region of the *ermB* gene on Tn*917* or Tn*1545*, and the regions found in macrolide-resistant *S. pneumoniae* in this study. SD1 and SD2, ribosome-binding sites for the leader peptide and *ermB* gene, respectively; −10 and −35, putative promoter regions according to references 7 and 8. Deletions are indicated by dashes.

TTCTGGTACTAAAAGTGG-3′ (18). Primers 5′-ATCTGACGGTGACATCT CTC-3′ and 5′-GGTTGAGTACCTTTTCATTCGTTAA-3′ for Tn*917* (17), and primers 5′-CTTAGAAGCAAACTTAAGAGTGTGT-3′ and 5′-GGTTGAGTA CCTTTTCATTCGTTAA-3 for Tn*1545* (22), were designed to amplify a fragment including the regulatory region and 48 bp of the 5′ end of *ermB*. PCR was carried out by 35 cycles of amplification consisting of 1 min of denaturation at 94°C, 1 min of annealing at 52°C, and 1 min of elongation at 72°C, followed by heating at 72°C for 7 min. PCR products were analyzed by using a DNA sequencing kit (ABI PRISM Big Dye Terminator cycle sequencing, FS; Perkin-Elmer Applied Biosystems, Tokyo, Japan). *S. pneumoniae* BM4200 containing Tn*1545*, kindly provided by P. Courvalin (Institut Pasteur, Paris, France) (22), and *E. faecalis* DS16 containing Tn*917* (17), kindly provided D. B. Clewell (University of Michigan, Ann Arbor, Mich.), were used as positive controls. Reaction products were precipitated with ethanol-potassium acetate, dissolved in template suppression reagent, and run on an ABI PRISM 310 sequencer. As a result, the organisms were separated into two groups: a Tn*917* group and a Tn*1545* group.

Analysis of reduction of telithromycin susceptibility by disk diffusion. The disk diffusion test was performed (9) using six strains selected from both groups based on the MICs for erythromycin and rokitamycin. Against the six strains, the MICs of erythromycin and rokitamycin were both >128 mg/liter (two strains), 4 and 8 mg/liter (two strains), and 4 and 1 mg/liter (two strains), respectively. Two milliliters of the bacterial suspension (approximately 10^8 CFU/ml) was spread over the surface of 10 ml of sensitivity test agar containing 8% SHS. After excess bacterial suspension was removed, paper disks (diameter, 8 mm; Tokyo Roshi Kaisha, Tokyo, Japan) impregnated with erythromycin (20 μg/disk), azithromycin (20 μg/disk), rokitamycin (20 and 100 μg/disk), or telithromycin (10 μg/disk) were placed on the surface of each agar plate. Then the plates were incubated overnight at 35°C. The presence of induced telithromycin resistance was assessed based on the shape of the zone of inhibition around the telithromycin disk (whether the so-called D-shape was observed).

Serogroup and genotyping analysis. The isolates were cultured in Mueller-Hinton broth (Eiken Chemical Co.) with 8% SHS at 35°C for 18 to 24 h; they were then harvested by centrifugation at 3,000 × *g* for 10 min. The serogroup of each strain was determined by using antisera for *S. pneumoniae* (Seiken Antisera; Denkaseiken, Tokyo, Japan). The isolates were genotyped by pulsed-field gel electrophoresis (PFGE), as reported previously (23), after strains from both groups were selected based on the serogroup and the geographical location of isolation in addition to the MIC for the macrolides.

Nucleotide sequence accession number. The nucleotide sequence data obtained in this study are available in the GenBank/EMBL nucleotide database under accession number AB111455.

RESULTS

PCR-based detection of resistance genes and DNA sequence analysis. Of the 84 isolates tested, all possessed the *ermB* gene and none had the *mefA* gene. There were 65 strains in the Tn*917* group and 19 in the Tn*1545* group. After selection of representative strains, comprising 19 strains from the Tn*917* group and all strains from the Tn*1545* group, with high (>128 mg/liter) or low (<4 mg/liter) erythromycin and rokitamycin MICs, the regulatory region and *ermB* gene were analyzed and aligned with the same region of *ermB* previously reported in Tn*917* and Tn*1545* (Fig. 1).

It was found that the sequences of the Tn*1545* group isolates all were identical to that reported for Tn*1545* (22). In the Tn*917* group, the sequences of 19 strains were identical to that

TABLE 1. In vitro activities of macrolides, clindamycin, telithromycin, and penicillin G against macrolide-resistant *S. pneumoniae* strains with different transposons

Group	MIC (mg/liter)[a] of:					
	Erythromycin	Azithromycin	Rokitamycin	Clindamycin	Telithromycin	Penicillin G
Tn1545 group (n = 19)						
Range	≤0.06–>128	0.25–>128	≤0.06–128	≤0.06–>128	≤0.06–>0.25	≤0.06–1
MIC_{50}	**4**	>128	**1**	128	≤0.06	0.25
MIC_{90}	128	>128	**2**	>128	0.13	0.5
Tn917 group (n = 65)						
Range	0.5–128	1–>128	2–>128	16–>128	≤0.06–0.25	≤0.06–1
MIC_{50}	**128**	>128	**32**	>128	≤0.06	≤0.06
MIC_{90}	>128	>128	**>128**	>128	0.25	0.5

[a] MIC_{50} and MIC_{90}, MICs at which 50 and 90% of isolates, respectively, were inhibited. Boldfaced values indicate prominent differences between the two groups.

reported for Tn*917* (17), but two mutations were found. The first mutation was insertion of a TAAA motif at T591 (T421 in our data) (14, 24). Since the TAAA motif is recognized as a stop codon, the putative leader peptide was shortened by 9 amino acids. The second mutation was the deletion of 182 bp from T251 to T432 (T81 to T262 in our data) (17). The −35 region of the putative promoter was included in this deletion. But the *ermB* gene was expressed, as evidenced by the fact that the strains with Tn*917* were resistant to macrolides and clindamycin. This finding suggests the presence of a new promoter.

Susceptibility. The MIC data for the macrolide-resistant *S. pneumoniae* strains with different transposons are displayed in Table 1. The MICs of erythromycin and rokitamycin were higher for strains from the Tn*917* group than for strains from the Tn*1545* group. In the Tn*1545* group, the MICs of erythromycin and clindamycin for two strains with the *ermB* gene were ≤0.25 mg/liter, but other strains exhibited the MLS_B phenotype. All of the isolates in the Tn*917* group had the MLS_B phenotype. Both groups showed resistance to azithromycin but were sensitive to telithromycin.

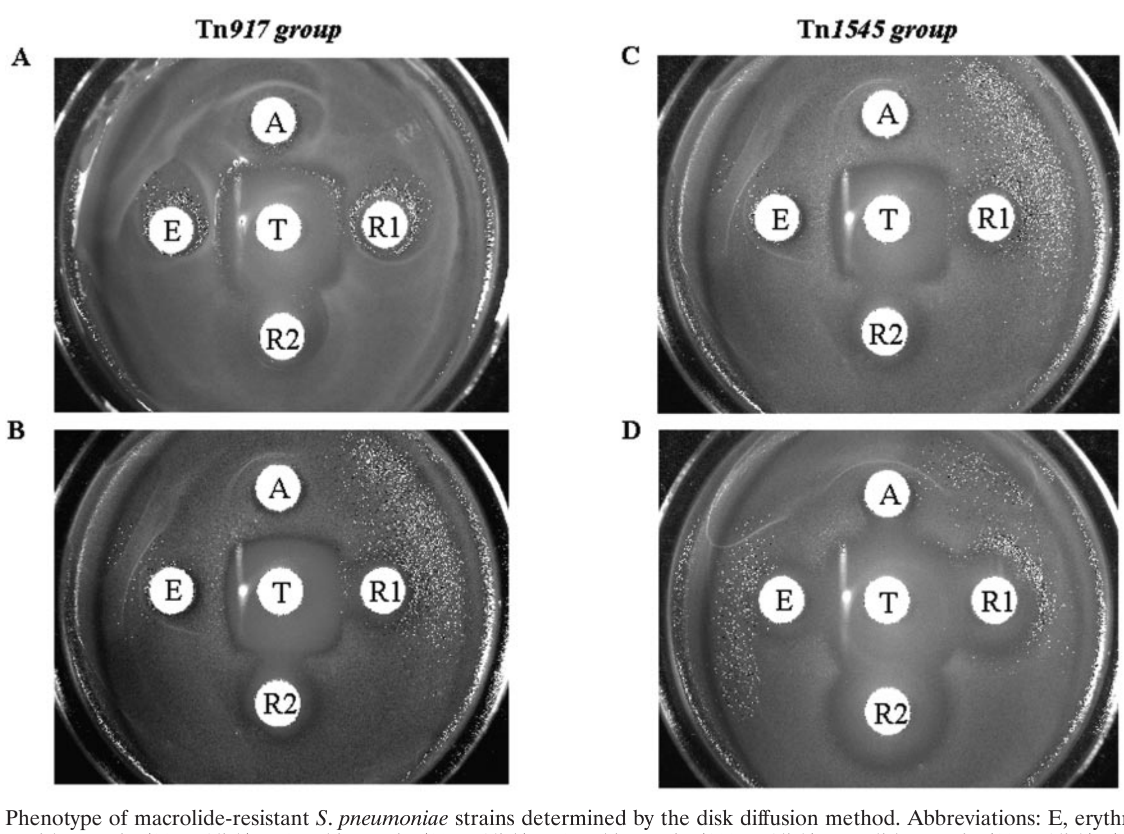

FIG. 2. Phenotype of macrolide-resistant *S. pneumoniae* strains determined by the disk diffusion method. Abbreviations: E, erythromycin (20 μg/disk); A, azithromycin (20 μg/disk), R1, rokitamycin (20 μg/disk); R2, rokitamycin (100 μg/disk); T, telithromycin (10 μg/disk). (A) MICs (in milligrams per liter) were >128 for E, A, and clindamycin; 128 for R; and 0.25 for T. (B) MICs (in milligrams per liter) were 4 for E, >128 for A and clindamycin, 8 for R, and 0.13 for T. (C) MICs (in milligrams per liter) were >128 for E, A, and clindamycin; 128 for R; and 0.25 for T. (D) MICs (in milligrams per liter) were 4 for E, >128 for A, 1 for R, 128 for clindamycin, and 0.06 for T.

TABLE 2. The five most frequent serogroups in each group of macrolide-resistant *S. pneumoniae* strains

Strains	No. (%) of isolates belonging to the following serogroup:					
	3	6	14	19	23	Others
Tn*1545* group (n = 19)	2 (10.5)	12 (63.2)	1 (5.3)	0 (0)	2 (10.5)	2 (10.5)
Tn*917* group (n = 65)	19 (29.2)	8 (12.3)	5 (7.7)	4 (6.2)	15 (23.1)	14 (21.5)

Reduction in telithromycin susceptibility. All of the strains tested showed a reduction in telithromycin susceptibility, and an inhibition zone was created by erythromycin, azithromycin, and rokitamycin. In the Tn*917* group, a square zone of inhibition was similarly formed regardless of whether erythromycin and rokitamycin MICs were high or low (Fig. 2A and B). In the Tn*1545* group, the strains with high erythromycin and rokitamycin MICs formed a square inhibition zone like that seen in the Tn*917* group, but inhibition was recognized more clearly around the rokitamycin disk (20 or 100 μg/disk), and the zone of inhibition was larger, if the erythromycin and rokitamycin MICs for the isolates were low (Fig. 2C and D).

Serogroup and genotype. Serogroup data are shown in Table 2. In the Tn*1545* group, 12 isolates (63.2%) belonged to serogroup 6. In the Tn*917* group, 19 strains (29.2%) and 15 strains (23.1%) belonged to serogroups 3 and 23, respectively, while 43 strains (47.7%) belonged to other serogroups.

PFGE results are shown in Fig. 3. In 17 strains of the Tn*1545* group, the same pattern was observed when isolates from a single geographic region were tested (Fig. 3A; Table 3). In 36 strains of the Tn*917* group, however, only a few strains showed similar patterns and most were quite different, even though the strains were isolated in the same geographical region (Fig. 3B; Table 3). These findings support the concept that Tn*1545* was spread clonally while Tn*917* was spread both clonally and horizontally.

DISCUSSION

We assessed characteristics of the macrolide resistance gene (*ermB*) in 84 clinical isolates of *S. pneumoniae* obtained from Japanese patients. The rate of macrolide resistance among *S. pneumoniae* strains isolated in Japan is 77.9%, a rate similar to that in Hong-Kong or South Korea, and 52.7% of macrolide-resistant strains possess the *ermB* gene (6). Thus, strains showing high resistance to macrolides are widespread in Japan.

In this study, the major macrolide resistance element in Japanese isolates was Tn*917* (or a Tn*917*-like element). Although it is not clear why Tn*917* is spreading among Japanese *S. pneumoniae* strains, it is possible that strains with Tn*917* are more resistant to 16-membered-ring macrolides than isolates with Tn*1545*, since macrolides with a 16-membered-ring (such as rokitamycin) are widely used in Japan.

We found the deletion of 182 bp of the Tn*917* sequence, including the −35 region of the *ermB* promoter, in all strains with Tn*917* isolated in Japan. Although the existence of a new promoter is conceivable, it could not be determined by computer simulation because we did not find a new promoter that was stronger than the putative original promoter. It is thought that these mutations have contributed to the increase in the production of the methylase, but those mechanisms have not been clarified. Although Oh et al. found a relationship between

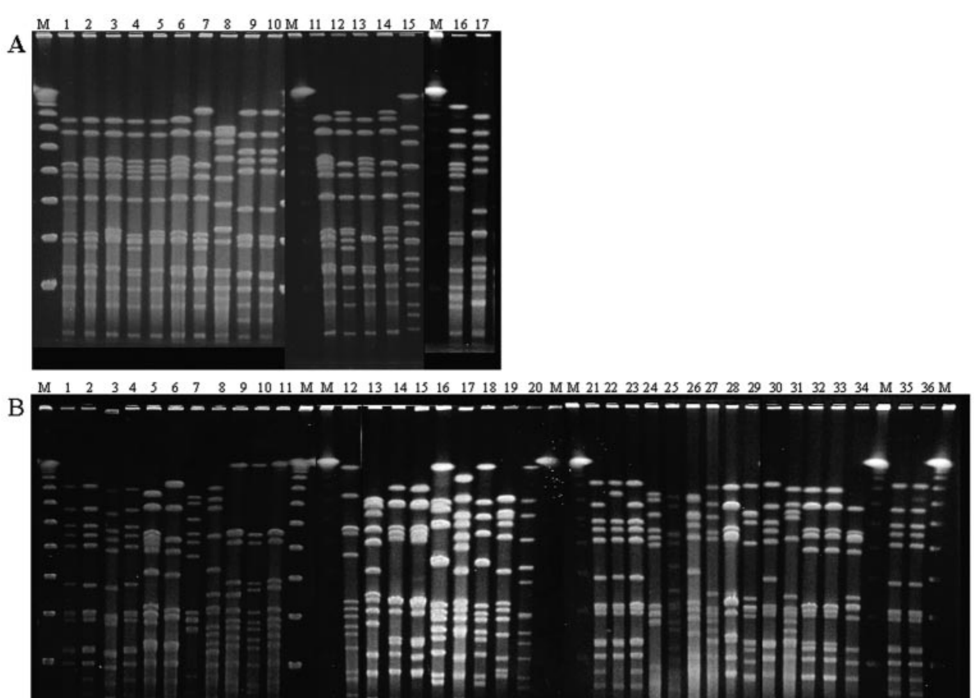

FIG. 3. PFGE of *S. pneumoniae* chromosomal DNA after digestion with the SmaI restriction enzyme. (A) Strains with Tn*1545*. (B) Strains with Tn*917*. Lanes M, lambda ladder. For details, see Table 3.

TABLE 3. PFGE results[a] for macrolide-resistant *S. pneumoniae*

Panel	Lane	Strains	Serogroup	Geographical region of isolation
A	1	KU5032	6	Kitasato
	2	KU5012	6	Kitasato
	3	KU3901	6	Kitasato
	4	KU4994	6	Kitasato
	5	KU3900	6	Kitasato
	6	KU4989	6	Kitasato
	7	KU5017	6	Kitasato
	8	KU3915	6	Kitasato
	9	KU5008	3	Kitasato
	10	KU4997	3	Kitasato
	11	B4	6	Sendai
	12	B1	6	Sendai
	13	B3	6	Sendai
	14	B5	6	Sendai
	15	A5	Other	Sendai
	16	TK9	14	Takahagi
	17	TK11	23	Takahagi
B	1	KU5197	3	Kitasato
	2	KU3784	3	Kitasato
	3	KU3781	3	Kitasato
	4	KU5019	3	Kitasato
	5	KU5003	6	Kitasato
	6	KU3800	6	Kitasato
	7	KU5213	14	Kitasato
	8	KU5020	14	Kitasato
	9	KU3791	23	Kitasato
	10	KU3785	23	Kitasato
	11	KU5203	23	Kitasato
	12	A12	6	Sendai
	13	A3	19	Sendai
	14	A13	23	Sendai
	15	A7	23	Sendai
	16	A4	Other	Sendai
	17	A2	Other	Sendai
	18	A9	Other	Sendai
	19	A8	Other	Sendai
	20	B2	Other	Sendai
	21	NU32	3	Nagasaki
	22	NU38	3	Nagasaki
	23	NU59	3	Nagasaki
	24	NU30	14	Nagasaki
	25	NU16	14	Nagasaki
	26	NU67	19	Nagasaki
	27	NU73	19	Nagasaki
	28	NU21	23	Nagasaki
	29	NU44	23	Nagasaki
	30	TK3	3	Takahagi
	31	TK1	19	Takahagi
	32	TK8	23	Takahagi
	33	TK16	23	Takahagi
	34	TK15	23	Takahagi
	35	KSM3	3	Kashima
	36	KSM4	3	Kashima

[a] Details of the experiment for which results are shown in Fig. 3, by panel and lane.

macrolide resistance and two mutations of the leader peptide in the *ermB* gene on Tn*917* from *E. faecalis* by a reporter gene assay (14), they concluded that TAAA duplication at the T591 (T421 in our data) site (which was also detected in this study) generated a translation stop codon and shortened the leader peptide by 9 amino acids so that reporter gene expression was dramatically elevated, i.e., TAAA duplication increased expression of the methylase and resulted in strong resistance to rokitamycin.

Strains that had the *ermB* gene on either transposon (or on a transposon-like sequence) showed reductions in telithromycin susceptibility in the disk diffusion test. However, for strains with Tn*1545* for which the rokitamycin MIC was lower, less telithromycin resistance was reduced by the 100-μg rokitamycin disk than for strains having Tn*917* or Tn*1545* for which rokitamycin MICs were high. In contrast, the isolates with Tn*917* showed reductions in telithromycin susceptibility induced by the 100-μg rokitamycin disk irrespective of the rokitamycin MICs. Such a result cannot be explained clearly. It has been reported that the basal level of ribosomal methylation differs from strain to strain and that the nucleotide sequence of the regulatory region shows variation in each isolate, resulting in different levels of constitutive *ermB* gene expression (24). However, we found that all strains had the same leader sequence of the *ermB* gene, even if the macrolide MICs were different. Such differences may be specific to each strain and could be based on complex mechanisms (11).

Although all strains were initially sensitive to telithromycin, the susceptibility of telithromycin was decreased by erythromycin, azithromycin, and rokitamycin (9). Because these macrolides are not used together clinically, this effect may not be a problem. In addition, there were no strains with constitutive macrolide resistance.

The PFGE patterns and serogroup distribution showed that Tn*917* spread both horizontally and clonally, while Tn*1545* spread clonally. The results of this study suggest that Tn*917* may have been transmitted from *E. faecalis* or other organisms, so that the dominant macrolide resistance gene is *ermB* on Tn*917* in Japanese pneumococci.

Recently, other mechanisms of macrolide resistance (amino acid substitutions in domain V of 23S rRNA or mutations of L4 and L22 proteins) in vitro (3, 20) and in clinical isolates (2, 15, 21) have been reported often. Kaieda et al. reported an *S. pneumoniae* strain with an *ermB* mutation that showed in vitro constitutive macrolide resistance (S. Kaieda, S., H. Yano, N. Okitsu, Y. Hosaka, R. Okamoto, H. Takahashi, and M. Inoue, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-1580, p. 72, 2002). It was isolated along with inducible macrolide resistance strains by culture with a disk containing 0.5 mg of telithromycin/liter, and the MIC for telithromycin was 8 mg/liter. However, the isolation rate was very low (10^{-10}), so it is thought that the appearance of mutants with constitutive resistance to macrolides, including telithromycin, is very uncommon in the clinical setting.

It is important to continue the surveillance of antimicrobial susceptibility in *S. pneumoniae* and to carefully monitor the emergence of constitutive macrolide resistance and resistance due to ribosomal mutations in *S. pneumoniae*.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the COE program of the Japanese Ministry of Science, Education, and Culture and a grant from the Japanese Ministry of Health, Sports, Science, and Technology (H12-Sinko-19).

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